Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 3599–3603, May 1989
Biochemistry

Isolation and characterization of a mammalian gene encoding a high-affinity cAMP phosphodiesterase

(neurobiology/oncogenes/yeast expression vectors/polymerase chain reaction)

JOHN COLICELLI, CARMEN BIRCHMEIER, TAMAR MICHAELI, KATHY O'NEILL, MICHAEL RIGGS, AND MICHAEL WIGLER*

Cold Spring Harbor Laboratory, P.O. Box 100, Cold Spring Harbor, NY 11724

Communicated by James D. Watson, February 13, 1989

ABSTRACT **A rat brain cDNA library has been constructed in a *Saccharomyces cerevisiae* expression vector and used to isolate genes that can function in yeast to suppress the phenotypic effects of *RAS2*^{val19}, a mutant form of the *RAS2* gene analogous to an oncogenic mutant of the human *HRAS* gene. One cDNA, DPD, was cloned and its genetic and biochemical properties were characterized. A DPD product would share 80% amino acid sequence identity with the *Drosophila melanogaster dunce*-encoded protein over an extended region. We have shown that the DPD protein is a high-affinity cAMP-specific phosphodiesterase.**

The yeast *Saccharomyces cerevisiae* encodes two genes, *RAS1* and *RAS2*, that have structural and functional homology with mammalian *RAS* oncogenes (1–4). When an activated form of the *RAS2* gene (*RAS2*^{val19}) is present, yeast cells fail to synthesize glycogen, are unable to arrest in G_1, are intolerant of nutrient starvation, and are acutely sensitive to heat shock (5, 6). These phenotypes, collectively referred to as loss of growth control, are primarily the result of overexpression or uncontrolled activation of the cAMP effector pathway via adenylyl cyclase (2, 5, 7, 8).

We have previously reported the isolation of two yeast genes, *PDE1* and *PDE2*, the low- and high-affinity cAMP phosphodiesterase-encoding genes, respectively, by their ability to suppress the heat shock sensitivity in yeast cells harboring an activated *RAS2*^{val19} gene (6, 9). We now report the use of a rat brain cDNA library to clone a mammalian cDNA that is able to complement the loss of growth control associated with this activated *RAS2* gene in yeast.† The gene, *DPD* (dunce-like phosphodiesterase), encodes a high-affinity cAMP phosphodiesterase that is highly homologous to the cAMP phosphodiesterase encoded by the *dunce* locus of *Drosophila melanogaster*. *D. melanogaster* with mutations in *dunce* have learning and memory defects (10, 11).

MATERIALS AND METHODS

Strains, Media, Transformations, and Heat Shock. *Escherichia coli* strain HB101 was used for plasmid propagation and isolation, and strain SCS1 (Stratagene) was used for transformation and maintenance of the cDNA library (12, 13). *S. cerevisiae* strains TK161-R2V (*MAT***a** *leu2 his3 ura3 trp1 ade8 can1 RAS2*^{val19}) (5) and 10DAB (*MAT*α *leu2 his3 ura3 ade8 pde1::ADE8 pde2::URA3 ras1::HIS3*) were used. 10DAB was created from a segregant of a diploid strain produced by mating TS-1 (14) and DJ23-3C (15). The segregant (*MAT*α *leu2 his3 ura3 ade8 pde1::LEU2 pde2::URA3 ras1::HIS3*) was subsequently transformed with the 5.4-kilobase-pair (kbp) *Xba* I *pde1::ADE8* fragment of pYT19-DAB to yield strain 10DAB. Yeast cells were grown in either rich medium (YPD, yeast extract/peptone/dextrose) or synthetic medium with appropriate auxotrophic supplements (SC) (16). Transformation of yeast cells was performed with lithium acetate (17). Heat shock experiments were performed by replica plating onto preheated SC plates that were maintained at 55°C for 10 min, allowed to cool, and incubated at 30°C for 24–48 hr. Segregation analysis was performed by growing yeast transformants in YPD for 2–3 days, plating onto YPD plates, and replica plating onto YPD, SC-leucine (plasmid selection), and YPD heat shock plates.

Plasmids, DNA Manipulations, and Sequencing. Plasmid DNA from individual *E. coli* colonies was purified by standard procedures (18, 19). Extrachromosomal DNA was isolated from yeast as described (9). The plasmid pYT19DAB was constructed from pYT19 (9) by first deleting *PDE1* sequences between the *Sma* I and *Bal* I restriction sites to yield pYT19D. The 4-kbp *Bam*HI fragment of the *ADE8* gene was then inserted into the *Bam*HI site of pYT19D to yield pYT19DAB. The cloning vector pADNS is based on the plasmid pAD1 previously described (20). pADNS consists of a 2.2-kbp *Bgl* II/*Hpa* I fragment containing the *S. cerevisiae* *LEU2* gene from YEp213 (21), a 1.6-kbp *Hpa* I/*Hin*dIII fragment of the *S. cerevisiae* 2-μm plasmid containing the origin of replication, and a 2.1-kbp *Ssp* I/*Eco*RI fragment containing the ampicillin-resistance gene from the plasmid pUC18. It also contains a 1.5-kbp *Bam*HI/*Hin*dIII fragment of the modified *S. cerevisiae* alcohol dehydrogenase (*ADH1*) promoter (22, 23) and a 0.6-kbp *Hin*dIII/*Bam*HI fragment containing the *ADH1* terminator sequences. The promoter and terminator sequences are separated by a polylinker that contains the restriction endonuclease sites *Not* I, *Sac* II, and *Sfi* I between the existing *Hin*dIII and *Sac* I sites. The oligonucleotides used to create these sites were 5′-GGCCAAAAAGGCCGCGGCCGCA and 5′-TCGACCGGTTTTTCCGGCGCCGGCGTTCGA. The plasmid pADPD is a pADNS-derived plasmid containing the 2.17-kb *DPD* cDNA insert.

Sequencing was performed by the dideoxynucleotide chain-termination method (24, 25). GENALIGN was used to align the *DPD* and *dunce* sequences (GENALIGN is a copyrighted software product of IntelliGenetics; developed by Hugo Martinez). RNA was purified from Sprague–Dawley rat brains by published procedures (26–28). cDNAs were ligated to the *Not* I linker oligonucleotides 5′-AAGCGGCCGC and 5′-GCGGCCGCTT. The cDNAs were cleaved with *Not* I and cloned into the *Not* I site of pADNS by standard procedures.

Polymerase chain reactions (PCRs) were carried out in a thermocycler (Perkin–Elmer/Cetus) using a modification of

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: PCR, polymerase chain reaction.

*To whom reprint requests should be addressed.

†The sequence reported in this paper is being deposited in the EMBL/GenBank data base (accession no. J04563).

published procedures (29). Reaction mixtures contained template DNA (1 ng of cloned DNA or 1 μg of total first strand cDNA), 25 pmol of oligonucleotide primers, 200 μM deoxyribonucleotide triphosphates, 10 mM Tris·HCl (pH 8.4), 50 mM KCl, 3 mM $MgCl_2$, and 0.01% (wt/vol) gelatin. The oligonucleotide primers used, as designated in Fig. 3, were as follows: A, 5′-CACCCTGCTGACAAACCT[44]; B, 5′-ATGGAGACGCTGGAGGAA[153]; C, 5′-ATACGCCACATCAGAATG[676]; D, 5′-TACCAGAGTATGATTCCC[1449]; E, 5′-GTGTCGATCAGAGACTTG[1668]; F, 5′-GCACACAGGTTGGCAGAC[2048]. The numbers indicate position coordinates in Fig. 2. Primers C, E, and F are noncoding strand sequences. Thirty cycles (1.5 min at 94°C, 3 min at 55°C, and 7 min at 72°C) were performed and the reaction products were analyzed by polyacrylamide gel electrophoresis.

Phosphodiesterase Assays. Yeast cells were grown at 30°C for 36 hr in 1-liter cultures of synthetic medium (SC-leucine). Cells were harvested and washed with buffer C (20 mM Mes/0.1 mM $MgCl_2$/0.1 mM EGTA/1 mM 2-mercaptoethanol), were resuspended in 30 ml of buffer C with 50 μl of 1 M phenylmethylsulfonyl fluoride, and were disrupted with a French press. The extracts were centrifuged at 1600 × *g* for 10 min and the supernatants were spun at 18,000 × *g* for 90 min (4°C). The supernatant was assayed for phosphodiesterase activity (6, 9). All the reaction mixtures contained Tris·HCl (pH 7.5) (100 mM), cell extract (50 μg of protein per ml), 5′-nucleotidase (20 ng/ml; Sigma), and Mg^{2+} (10 mM) (unless otherwise stated) and the indicated cyclic nucleotide concentrations. Assays for cGMP hydrolysis used 1.5 μM cGMP. Inhibition studies used 5 μM cAMP in the presence of various amounts of cGMP up to 500 μM. [^{3}H]cAMP and [^{3}H]cGMP were from NEN. Reaction mixtures were incubated for 10 min at 30°C and stopped with 5× stop solution (250 mM EDTA/25 mM AMP/100 mM cAMP).

RESULTS

A Mammalian Gene That Can Revert the Heat Shock Sensitivity of *RAS2*^{val19} Yeast. We have previously described the isolation of several yeast genes that when overexpressed on extrachromosomal yeast vectors are capable of suppressing the heat shock sensitivity exhibited by the *RAS2*^{val19}-expressing strain TK161-R2V (6, 9). We have now used the same selection to isolate mammalian genes that can function in yeast to render *RAS2*^{val19} cells resistant to heat shock. A rat brain cDNA library was produced and cloned into the yeast expression vector pADNS (Fig. 1). cDNAs were ligated to *Not* I linkers, cleaved with *Not* I restriction enzyme, and cloned into pADNS at the *Not* I site situated between the alcohol dehydrogenase promoter and termination sequences. The use of the rare cutting *Not* I obviated the need for restriction site methylases commonly used in cDNA cloning.

Approximately 1.5 × 10^5 independent cDNA inserts were contained in the library, with an average insert size of 1.5 kbp. DNA prepared from the cDNA expression library was used to transform the *RAS2*^{val19} yeast strain TK161-R2V. The 50,000 Leu$^+$ transformants obtained were subsequently tested for heat shock sensitivity. Only one transformant displayed heat shock resistance which was conditional upon retention of the expression plasmid. A plasmid, pADPD, was isolated from this transformant and the 2.17-kb *Not* I insert was analyzed by restriction site mapping (Fig. 1) and nucleotide sequencing (24, 25) (Fig. 2).

A large open reading frame of 562 codons was found. The first ATG, however, appears at codon 46 and a protein that initiates at this codon would have a predicted molecular mass of ≈60 kDa. A second shorter open reading frame, separated from the first by three stop codons but in the same frame as the principal coding region, contains 116 codons. The nucleotide sequence of the coding strand ends with a stretch of

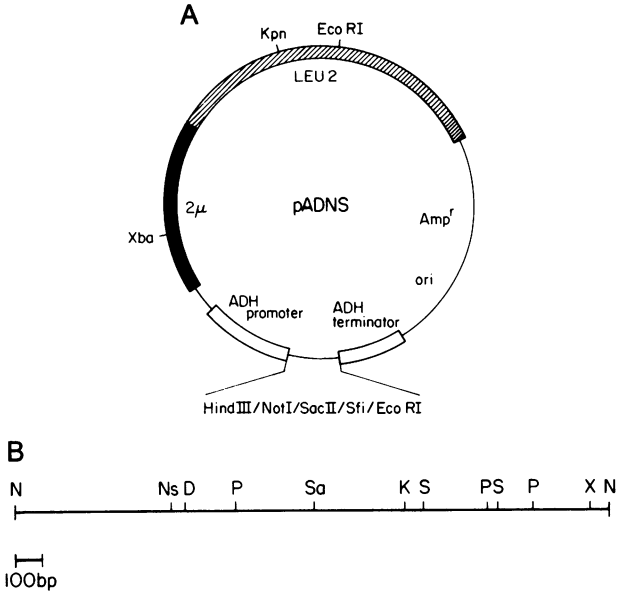

FIG. 1. Restriction maps of expression vector and isolated insert. (*A*) The expression vector pADNS is described in detail in *Materials and Methods*. It contains selectable markers for use in yeast (*LEU2*), and bacteria (*AMP*^{R}), as well as yeast and bacterial origins of replication. The yeast alcohol dehydrogenase sequences are shown with the *Not* I cloning site located between them. (*B*) The DPD cDNA insert is shown as a 2.17-kbp *Not* I fragment. N, *Not* I; Ns, *Nsi* I; D, *Dra* I; P, *Pvu* II; Sa, *Sac* I; K, *Kpn* I; S, *Stu* I; X, *Xba* I. The cDNA is presented with the coding strand oriented 5′ (left) to 3′ (right).

poly(A). A search for similar sequences was performed and the *D. melanogaster dunce* gene was found. The two genes would encode proteins with an 80% amino acid identity, without the introduction of gaps, over a 252-amino acid region located in the center of the rat DPD cDNA. The *dunce* gene has been shown to encode a high-affinity cAMP phosphodiesterase (30–32).

To demonstrate that the sequences upstream and downstream of the large sequence identity region were in fact contiguous with that region in the mRNA rather than artifacts of our method for cDNA cloning, we compared the structure of our cloned cDNA with DPD cDNAs contained in an independently prepared first strand cDNA population obtained by reverse transcribing total rat brain poly(A)$^+$ RNA with an oligo(dT) primer. Oligonucleotide primers complementary to sequences located within the identity region, and to sequences near the 5′ or 3′ ends of the coding strand, were made. Using either the cloned DNA or the total first-strand cDNA material as template, PCRs were carried out using four different primer sets and the reaction products were analyzed by polyacrylamide gel electrophoresis (Fig. 3). In each case, a fragment of the predicted length was obtained by using either of the template DNAs. The band assignments were confirmed by cleavage with restriction endonucleases having recognition sites within the amplified DNA product. Again, in each case, the primary PCR product obtained using either source of template yielded cleavage products of the predicted sizes. Some submolar background bands do appear in the PCR products but these were unaffected by the restriction digests. The results indicate that the sequence arrangement in the cloned cDNA faithfully reflects the structure of the rat mRNA.

Expression and Characterization of the *DPD* Gene Product. *S. cerevisiae* encodes two cAMP phosphodiesterase genes, *PDE1* and *PDE2* (6, 9). The strain 10DAB carries disruptions of both of these genes. The resulting cAMP phosphodiesterase deficiency leads to elevated intracellular cAMP

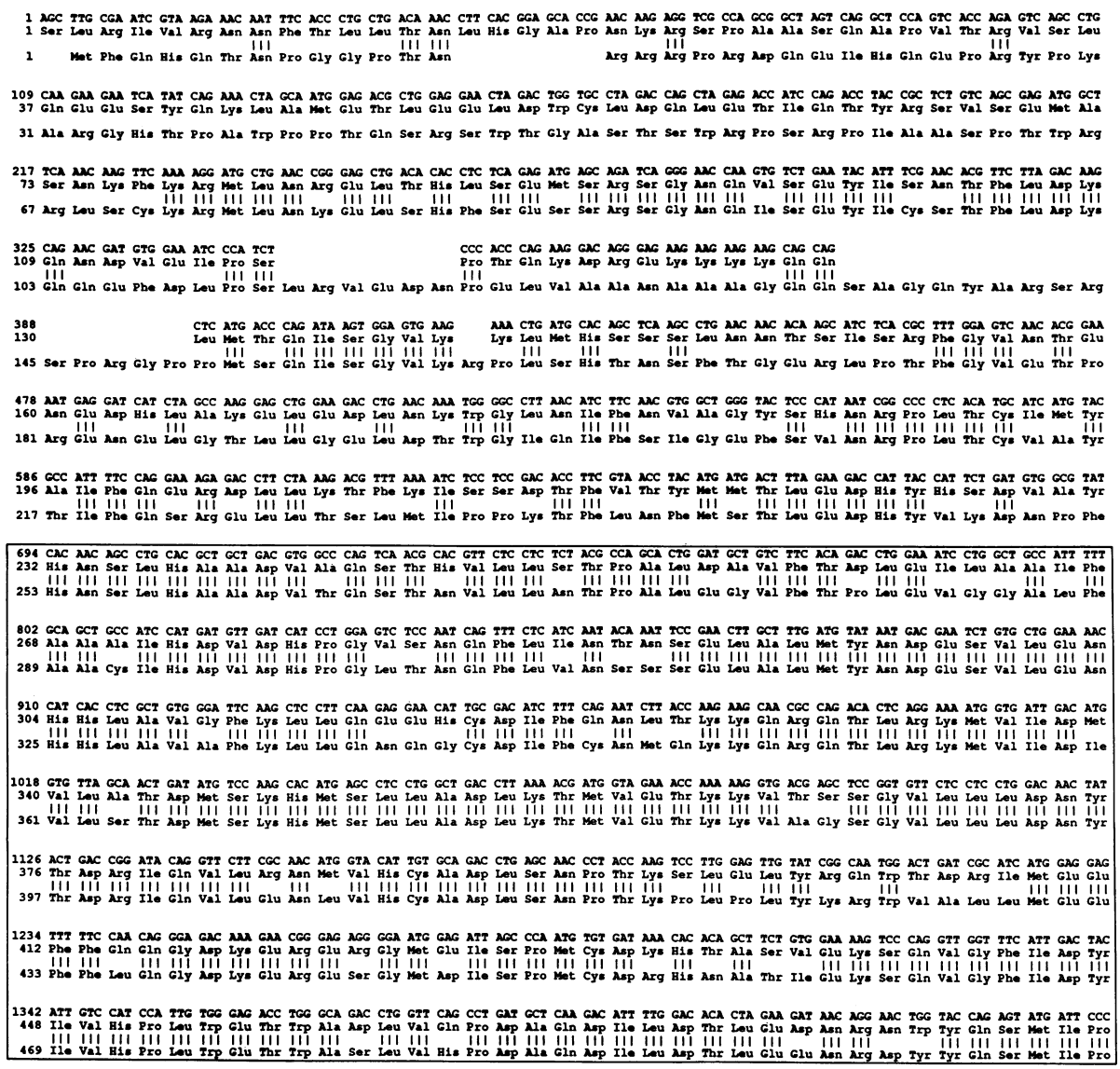

FIG. 2. The nucleotide sequence of the DPD cDNA. The top line shows the nucleotide sequence. Below is the predicted amino acid sequence of the open reading frame. Termination codons (indicated with a dot) are located at positions 563, 570, 575, and 692. The bottom row shows the amino acid sequence of the *Drosophila dunce* gene (28, 38). Identical amino acids in *DPD* and *dunce* are indicated with triple vertical lines. The area of greatest sequence identity is boxed. Numbers on the left indicate nucleotide and amino acid positions.

levels and a heat shock-sensitivity phenotype similar to that of strains harboring the *RAS2*^{val19} allele (9). 10DAB cells were transformed with the *DPD* expression plasmid pADPD, were assayed for heat shock sensitivity, and were indeed rendered resistant to heat shock (Fig. 4).

To analyze the biochemical properties of the *DPD* gene product, crude cell extracts were prepared from 1-liter cultures of 10DAB that had been transformed with either pADNS or pADPD. Phosphodiesterase activity assays were performed using cAMP as substrate. Control extracts

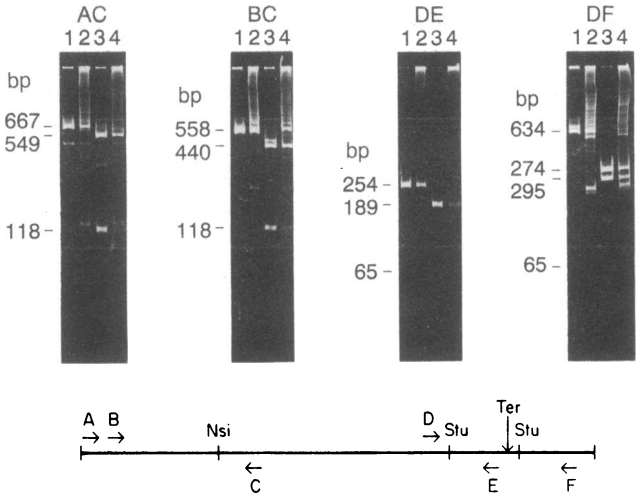

FIG. 3. Analysis of DPD cDNA structure using PCR. Reactions were carried out as described and the reaction products (either untreated or cleaved with a restriction endonuclease) were analyzed on a polyacrylamide gel stained with ethidium bromide. The diagram at the bottom of the figure illustrates the DPD cDNA and the positions of primer oligonucleotides (A, B, C, D, E, and F) used. The locations of the restriction sites for *Nsi* I and *Stu* I are also shown, as is the position of the termination codons (Ter). Each of the four panels is labeled to indicate the primers used for PCR. Lanes: 1, PCR product using the cloned DNA as template; 2, PCR product resulting from the single-stranded cDNA template made from total rat brain poly(A)$^+$ RNA; 3 and 4, PCR products from the cloned DNA template or cDNA template, respectively, which have been cleaved with *Nsi* I (AC and BC) or *Stu* I (DE and DF). Restriction fragment lengths are indicated on the left. These lengths have been calculated from the known sequence and are in agreement with the observed mobility of standard DNA fragments run on the same gel.

(10DAB with pADNS) showed no cAMP phosphodiesterase activity. Results with the controls were unchanged when performed at 0°C or in the absence of Mg^{2+} and were comparable to results obtained when no extract was added. These results indicate that there is indeed no detectable background phosphodiesterase activity in this strain.

In contrast, considerable cAMP phosphodiesterase activity was seen in the 10DAB strain transformed with pADPD. The rate of cAMP hydrolysis in cells containing *DPD* was measured as a function of cAMP concentration (Fig. 5). The deduced K_m for cAMP is 3.5 μM and the calculated V_{max} is 1.1 nmol·mg^{-1}·min^{-1}.

The assay conditions were varied to ascertain the cation preferences of the enzyme and to determine the ability of calcium and calmodulin to stimulate its activity. In these assays, Mn^{2+} can be used as well as Mg^{2+}, and either cation

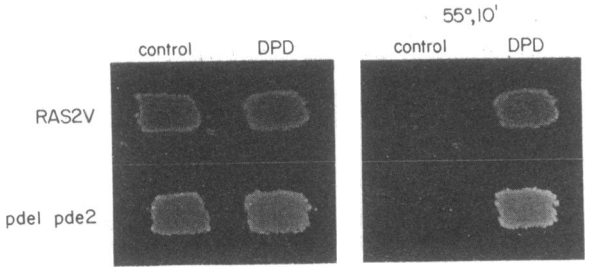

FIG. 4. Heat shock phenotype. Two heat shock-sensitive yeast strains, TK161-R2V (*RAS2*^{val19}) and 10DAB (*pde1*$^-$, *pde2*$^-$), were transformed with either the pADNS cloning vector alone (control), or with the pADPD plasmid expressing the rat brain phosphodiesterase (*DPD*). Yeast patches were grown on synthetic medium plates for 1 day and then replica plated to fresh plates at 30°C (*Left*) or to preheated plates and incubated at 55°C for 10 min before returning to 30°C (*Right*). Recovery time at 30°C was 36 hr.

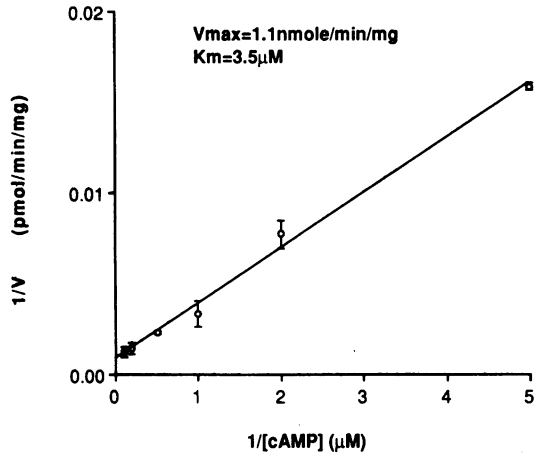

FIG. 5. DPD enzyme kinetics. Phosphodiesterase assays were performed on cell extracts as described with a final Mg^{2+} concentration of 10 mM. The cAMP concentration was varied from 0.2 to 10 μM. Two independent determinations were made and background measured in vector-only extracts was subtracted. Error bars are based on SD.

in 1 mM final concentration was sufficient. Calcium/calmodulin was unable to stimulate the measured phosphodiesterase activity in the extract (data not shown). A parallel assay in which beef heart phosphodiesterase (Boehringer Mannheim) was used yielded a 6.5-fold stimulation with the addition of calcium/calmodulin (data not shown). Finally, no cGMP phosphodiesterase activity was detected in our assays. Beef heart phosphodiesterase was again used as a positive control. In addition, cGMP present in amounts 100-fold over substrate concentrations was unable to inhibit cAMP phosphodiesterase activity.

DISCUSSION

Previous workers have cloned a mammalian gene in yeast by using a biological screen (33). In that case, a homolog to the *cdc2* gene of *Schizosaccharomyces pombe* was cloned by screening a cDNA library for complementation of *cdc2* mutants. In that library, the cDNAs were inserted proximal to the simian virus 40 early large tumor antigen promoter. In our work, we have used a library with mammalian cDNAs inserted into a yeast expression vector, proximal to a strong yeast promoter. In addition, we have used *Not* I linkers for cDNA cloning, which allows the convenient subcloning of an entire insert library from one vector to another. We feel that this will be a generally useful approach for cloning genes from higher eukaryotes when functional screens are possible in yeast. This system is particularly useful for the cloning of other cAMP phosphodiesterases from mammals. The availability of yeast strains totally lacking endogenous cAMP phosphodiesterase activity will also facilitate the biochemical characterization of these new phosphodiesterases.

The mammalian DPD cDNA can encode a protein with a high degree of amino acid sequence identity (80%) with the predicted *D. melanogaster dunce* gene product over an extended region. The *dunce* gene has been shown to encode a high-affinity cAMP phosphodiesterase required for normal learning and memory in flies (30–32). Compared to the striking level of sequence identity between *DPD* and *dunce*, the sequence conservation among other known cAMP phosphodiesterases is scant (34). Therefore, the *DPD–dunce* homology in the conserved region represents more than a constraint on sequences required for cAMP binding and hydrolysis and suggests a conservation of interactions with other components.

Biochemical characterization of the DPD cDNA product expressed in yeast indicates that it is a high-affinity cAMP-specific phosphodiesterase, as is *dunce* (31, 32). In addition, *DPD* activity, as measured in our assays, is not stimulated by the presence of calcium/calmodulin. This property is shared with *dunce* and is distinct from some other phosphodiesterases (for a review, see ref. 35). The two proteins, DPD and dunce, thus appear to have similar biochemical characteristics. However, it should also be noted that *DPD* encodes a protein product that shows much less significant homology (35%) to *dunce* beyond the previously described highly conserved region. These nonconserved sequences could result in an altered or refined function for this mammalian *dunce* homolog.

Since the predicted rat DPD product diverges from the *Drosophila dunce* gene product, and since we have merely a single cDNA isolate, we were concerned that the structure of our cDNA might not reflect the structure of the DPD mRNA. We have described here the use of PCRs to compare the structure of our DPD cDNA with the DPD mRNA. This study indicates a complete concordance in structure. Our method should also be applicable to the detection and analysis of alternate mRNA splicing (see below).

Our *DPD* sequence encodes a methionine codon at position 46 and the established reading frame remains open through to position 563, resulting in a protein with a predicted molecular mass of 60 kDa. The same reading frame, however, is open beyond the 5′ end of the coding strand (Fig. 2). At present, we cannot say whether the methionine codon at position 46 is the initiating codon for the DPD protein. The coding sequence is interrupted by three closely spaced terminator codons. However, the established reading frame then remains open for an additional 116 codons, followed by more terminator codons, a polyadenylylation consensus signal, and a poly(A) stretch. This 3′ open reading frame could be incorporated into another *dunce*-like phosphodiesterase through alternate splicing. To examine this, we utilized the PCR method by using oligonucleotides from the conserved region and from the downstream open reading frame [(oligo(D) and -(F), respectively, in Fig. 3]. Our PCR method reveals no evidence of a DPD mRNA in adult rat brain that utilizes both the highly conserved domain and the open reading frame 3′ to the stop codons. It should be noted, however, that a complex transcription pattern involving alternately spliced messages has been described for the *D. melanogaster dunce* locus (36, 37), and this may also be a feature of the mammalian homolog.

Davis *et al.* (38) have recently isolated a mammalian *dunce* homolog from a rat brain cDNA library by standard nucleic acid hybridization techniques. The gene they describe is indeed similar to, though distinct from, the DPD cDNA described here. Within the highly conserved region, as defined in this work, the predicted amino acid sequences of the two rat genes are 93% identical. This homology falls off dramatically, however, in the flanking regions, which show amino acid identities of 60% (upstream) and 30% (downstream) and require the use of sequence gaps for optimum alignment. These differences should be sufficient to distinguish the two related messages in *in situ* hybridizations and to permit the study of their distribution throughout the adult rat brain and during development.

We thank Ronald L. Davis for sharing his results prior to publication. We also thank Ilse Wieland and Kenneth Ferguson for technical advice. We also thank Patricia Bird for her help in preparation of this manuscript. This work was supported by grants from the National Institutes of Health, the Pfizer Biomedical Research Award, the American Cancer Society, and the American Business Foundation for Cancer Research. J.C. is a Schering-Plough Foundation Fellow of the Life Sciences Research Foundation, T.M. is supported by the Damon Runyon–Walter Winchell Cancer Fund, and M.W. is an American Cancer Society Professor.

1. Powers, S., Kataoka, T., Fasano, O., Goldfarb, M., Strathern, J., Broach, J. & Wigler, M. (1984) *Cell* **36**, 607–612.
2. Kataoka, T., Powers, S., McGill, C., Fasano, O., Strathern, J., Broach, J. & Wigler, M. (1984) *Cell* **37**, 437–445.
3. DeFeo-Jones, D., Scolnick, E. M., Koller, R. & Dhar, R. (1983) *Nature (London)* **306**, 707–709.
4. Dhar, R., Nieto, A., Koller, R., DeFeo-Jones, D. & Scolnick, E. (1984) *Nucleic Acids Res.* **12**, 3611–3618.
5. Toda, T., Uno, I., Ishikawa, T., Powers, S., Kataoka, T., Broek, D., Cameron, S., Broach, J., Matsumoto, K. & Wigler, M. (1985) *Cell* **40**, 27–36.
6. Sass, P., Field, J., Nikawa, J., Toda, T. & Wigler, M. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 9303–9307.
7. Kataoka, T., Broek, D. & Wigler, M. (1985) *Cell* **43**, 493–505.
8. Field, J., Nikawa, J., Broek, D., MacDonald, B., Rodgers, L., Wilson, I. A., Lerner, R. A. & Wigler, M. (1988) *Mol. Cell. Biol.* **8**, 2159–2165.
9. Nikawa, J., Sass, P. & Wigler, M. (1987) *Mol. Cell. Biol.* **7**, 3629–3636.
10. Dudai, Y., Jan, Y., Byers, D., Quinn, W. G. & Benzer, S. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 1684–1688.
11. Tempel, B. L., Bonini, N., Dawson, D. R. & Quinn, W. G. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 1482–1486.
12. Mandel, M. & Higa, A. (1970) *J. Mol. Biol.* **53**, 159–162.
13. Hanahan, D. (1983) *J. Mol. Biol.* **166**, 557–580.
14. Kataoka, T., Powers, S., Cameron, S., Fasano, O., Goldfarb, M., Broach, J. & Wigler, M. (1985) *Cell* **40**, 19–26.
15. Nikawa, J.-I., Cameron, S., Toda, T., Ferguson, K. M. & Wigler, M. (1987) *Genes Dev.* **1**, 931–937.
16. Mortimer, R. K. & Hawthorne, D. C. (1969) in *The Yeast*, eds. Rose, A. H. & Harrison, J. S. (Academic, New York), Vol. 1, pp. 385–460.
17. Ito, H., Fukuda, Y., Murata, K. & Kimura, A. (1983) *J. Bacteriol.* **153**, 163–168.
18. Holmes, D. S. & Quigley, M. (1981) *Anal. Biochem.* **114**, 193–197.
19. Katz, L., Kingsbury, D. T. & Helinski, D. E. (1973) *J. Bacteriol.* **114**, 477–485.
20. Powers, S., Michaelis, S., Broek, D., Anna, S. S., Field, J., Herskowitz, I. & Wigler, M. (1986) *Cell* **47**, 413–422.
21. Sherman, F., Fink, G. R. & Hicks, J. B. (1986) in *Laboratory Course Manual for Methods in Yeast Genetics*, eds. Sherman, F., Fink, G. R. & Hicks, J. B. (Cold Spring Harbor Lab., Cold Spring Harbor, NY), pp. 95–97.
22. Bennetzen, J. L. & Hall, B. D. (1982) *J. Biol. Chem.* **257**, 3018–3025.
23. Ammerer, G. (1983) *Methods Enzymol.* **101**, 192–201.
24. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
25. Biggin, M. D., Gibson, T. J. & Hong, G. F. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 3963–3965.
26. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. (1979) *Biochemistry* **18**, 5294–5299.
27. Lizardi, P. M. (1983) *Methods Enzymol.* **96**, 24–38.
28. Watson, C. J. & Jackson, J. F. (1984) in *DNA Cloning: A Practical Approach*, ed. Glover, D. (IRL, Oxford), pp. 79–88.
29. Saiki, R., Gelfand, D., Stoffe, S., Scharf, S., Higushi, R., Horn, G., Mullis, K. & Erlich, H. (1988) *Science* **239**, 487–491.
30. Chen, C., Denome, S. & Davis, R. L. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 9313–9317.
31. Davis, R. L. & Kiger, J. A. (1981) *J. Cell Biol.* **90**, 101–107.
32. Walter, M. F. & Kiger, J. A. (1984) *J. Neurosci.* **4**, 495–501.
33. Lee, M. G. & Nurse, P. (1987) *Nature (London)* **327**, 31–35.
34. Charbonneau, H., Beier, N., Walsh, K. A. & Beavo, J. A. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 9308–9312.
35. Beavo, J. A. (1988) in *Advances in Second Messenger and Phosphoprotein Research*, eds. Greengard, P. & Robinson, G. A. (Raven, New York), Vol. 22, pp. 1–38.
36. Davis, R. L. & Davidson, N. (1986) *Mol. Cell. Biol.* **6**, 1464–1470.
37. Chen, C., Malone, T., Beckendorf, S. K. & Davis, R. L. (1987) *Nature (London)* **329**, 721–724.
38. Davis, R. L., Takayasu, H., Eberwine, M. & Myres, J. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 3604–3608.